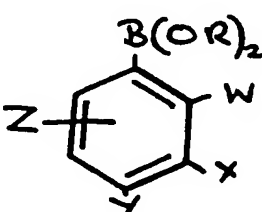




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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/GB93/00271 <b>(22) International Filing Date:</b> 10 February 1993 (10.02.93)  <b>(30) Priority data:</b> 9202769.7                      10 February 1992 (10.02.92)    GB 9216784.0                      7 August 1992 (07.08.92)        GB  <b>(71) Applicant:</b> BRITISH TECHNOLOGY GROUP LTD. [GB/GB]; 101 Newington Causeway, London SE1 6BU (GB).  <b>(72) Inventor:</b> KRICKA, Larry, Jan ; 886 Nathan Hale Road, Berwyn, PA 19312 (US).  <b>(74) Agent:</b> PERCY, Richard, Keith; Patents Department, Brit- ish Technology Group Ltd., 101 Newington Causeway, London SE1 6BU (GB).		<b>(81) Designated States:</b> AU, CA, FI, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.          Before the expiration of the time limit for amending the          claims and to be republished in the event of the receipt of          amendments.</i>
<b>(54) Title:</b> CHEMILUMINESCENT ENHANCERS  <div style="text-align: center;">  <p>(I)</p> </div> <b>(57) Abstract</b>  <p>A method of increasing the light output and/or signal: background ratio of light output from a chemiluminescent reaction of a dihydropthalazinedione (DPD), a peroxidase enzyme catalyst and an oxidant, by carrying out this reaction in the presence of an enhancer, "signal" being in the presence of the peroxidase, "background" in its absence, characterised in that the enhancer includes compounds of formula (I) in which R, W, X, Y and Z have the following meanings: R is selected from hydrogen, n-butyl, O,O-propylene (a cyclic ether), 4'-chlorophenyl and 3',5'-dichlorophenyl, W is selected from hydrogen, hydroxy, methyl, methoxy and chloro, X is selected from hydrogen, methyl, chloro, amino and nitro, Y is selected from hydrogen, methyl, carboxy, chloro, bromo, iodo, phenyl, phenoxy, 4'-chloroanilino, 4'-boronylphenyl, 4'-bromophenyl, 2'-carboxyethenyl and trimethylsilyl, Z is selected from hydrogen, 5-chloro, 5-bromo, 5-(3'-trifluoromethyl)phenylazo, or 6-chloro, W and X together may represent a fused benzene ring and X and Y together may represent a fused benzene ring substituted by hydroxy in the 6-position of the naphthalene ring numbering, provided that when R is hydrogen, W, X, Y, Z are each separately hydrogen; when R, W, X and Z are each hydrogen Y is selected from iodo, bromo, chloro, trimethylsilyl, phenoxy, phenyl, 4'-chloroanilino, methyl, 4'-boronylphenyl, 2'-carboxyethenyl; when Y is hydrogen and the Rs together represent O,O-propylene (a cyclic ether), X is hydrogen; when R, W and Z are each hydrogen, X and Y together represent a fused benzene ring substituted by hydroxy in the 6-position of the naphthalene ring numbering; when W, X and Z are each hydrogen, R is n-butyl and Y is bromo or 4'-bromophenyl; when W, X and Z are each hydrogen, R is 4'-chlorophenyl and Y is chloro; when W and Y are each hydrogen, R is 3', 5'-dichlorophenyl, Y is chloro and Z is 5-chloro; when W is methoxy, Z is 5-bromo; when W is hydroxy, Z is 5-(3'-trifluoromethyl)phenylazo; when W is chloro, X is chloro when Y is chloro, X is nitro or chloro; when Y is carboxy, X is nitro; when W and Y are each chloro and X is amino, Z is 6-chloro; and the compounds bis(catechol)borate, boroglycine, pentaerythritol borate, 4-(3'-borono-4'-hydroxyphenylazo)benzoic acid, diphenylisobutoxyborane, diphenylboronic anhydride, dimethylphenylboronic acid (substitution pattern not established) and the sodium salt of 3-nitrophenylboronic acid.</p>		

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CHEMILUMINESCENT ENHANCERSBackground of the invention1. Field of the invention

5       The present invention relates to an enhanced chemiluminescent reaction especially for use in a diagnostic assay, particularly immunoassay, and to a diagnostic kit for use in the assay. A chemiluminescent reaction is a chemical reaction which results in the emission of light. The  
10       luminescent emission is generally of sufficient duration to enable the light emitted to be detected or measured, and thereby to allow the detection or quantification of an analyte. The chemiluminescent reaction with which this invention is concerned is that between a 2,3-dihydro-1,4-phthalazinedione (DPD),  
15       especially luminol, with an oxidant, especially hydrogen peroxide, and a peroxidase enzyme, especially horseradish peroxidase, which catalyses the oxidation of the DPD by the oxidant. The oxidation is accompanied by emission of light.

2. Description of the prior art

20       Luminescent assays making use of the above-mentioned peroxidase-catalysed oxidation of a DPD include several types. This invention is concerned primarily with those in which the presence or amount of peroxidase is determined. It includes predominantly assays wherein horseradish peroxidase is  
25       conjugated to a ligand in order to label it and a luminescent reaction is used to detect or quantitate the label. This category includes ELISAs, competitive EIAs and nucleic acid hybridization assays, based on peroxidase labels. However, assays for measurement of free peroxidase, e.g. for analytical  
30       purposes, are also included.

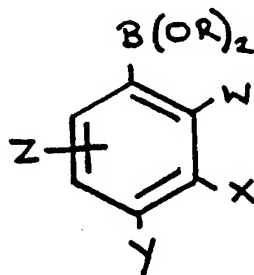
A review of luminescent assays has been published by L. J. Kricka, Clinical Chemistry 37, 1472-1481 (1991).

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The sensitivity of the peroxidase-catalysed chemiluminescent oxidation of DPDs can be enhanced by including in the reagents an enhancer, namely a 6-hydroxybenzothiazole (European Patent No. 87959B), a phenol selected from a narrowly defined class (European Patent No. 116454B or U.S. Patent No. 4,598,044), or an aromatic amine selected from a narrowly defined class (U.K. Patent No. 2162946B or U.S. Patent No. 4,729,950). A further class of substituted phenols that enhance chemiluminescent reactions of this type are phenols substituted in ortho and/or para positions by imidazolyl or benzimidazolyl (U.K. Patent No. 2205945B, European Patent No. 296752B). These patents are owned by National Research Development Corporation. European Patent Application Publication No. 219352A (Minnesota Mining and Mfg. Co.) describes various aromatic amines, including some of those previously mentioned in U.K. Application 2162946A, as enhancers. It is an object of the invention to extend the range of effective enhancers. This is a difficult task because no theory or mechanism has been published to explain how one should attempt to select candidate compounds to try as enhancers. For the purposes of the present application the term "enhancer" and related terms will be used to include compounds that increase the total light output and/or the signal:background ratio of a chemiluminescent assay, at at least one concentration of compound.

#### 25 Summary of the invention

It has now been found that certain organoboron compounds are effective enhancers of chemiluminescence in a reaction between a dihydrophthalazinedione (DPD), a peroxidase enzyme catalyst and an oxidant. The enhancers of the present invention include compounds of formula (I)



(I)

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in which R, W, X, Y and Z have the following meanings;

- R is selected from hydrogen, n-butyl, O,O-propylene (a cyclic ether), 4'-chlorophenyl and 3',5'-dichlorophenyl, W is selected from hydrogen, hydroxy, methyl, methoxy and chloro, X is selected from hydrogen, methyl, chloro, amino and nitro, Y is selected from hydrogen, methyl, carboxy, chloro, bromo, iodo, phenyl, phenoxy, 4'-chloroanilino, 4'-boronylphenyl, 4'-bromophenyl, 2'-carboxyethenyl and trimethylsilyl, Z is selected from hydrogen, 5-chloro, 5-bromo, 5-(3'-trifluoromethyl)phenylazo, or 6-chloro, W and X together may represent a fused benzene ring and X and Y together may represent a fused benzene ring substituted by hydroxy in the 6 position of the naphthalene ring numbering, provided that
- when R is hydrogen, W, X, Y, Z are each separately hydrogen;
- when R, W, X and Z are each hydrogen Y is selected from iodo, bromo, chloro, trimethylsilyl, phenoxy, phenyl, 4'-chloroanilino, methyl, 4'-boronylphenyl, 2'-carboxyethenyl;
- when Y is hydrogen and the Rs together represent O,O-propylene (a cyclic ether), X is hydrogen;
- when R, W and Z are each hydrogen, X and Y together represent a fused benzene ring substituted by hydroxy in the 6-position of the naphthalene ring numbering;
- when W, X and Z are each hydrogen, R is n-butyl and Y is bromo or 4'-bromophenyl;
- when W, X and Z are each hydrogen, R is 4'-chlorophenyl and Y is chloro;
- when W and Y are each hydrogen, R is 3',5'-dichlorophenyl, Y is chloro and Z is 5-chloro;
- when W is methoxy, Z is 5-bromo;
- when W is hydroxy, Z is 5-(3'-trifluoromethyl)phenylazo;
- when W is chloro, X is chloro;
- when Y is chloro, X is nitro or chloro;
- when Y is carboxy, X is nitro;

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when W and Y are each chloro and X is amino, Z is 6-chloro;  
 and the compounds bis(catechol)borate, boroglycine, penta-  
 erythritol borate, 4-(3'-borono-4'-hydroxyphenylazo)benzoic  
 acid, diphenylisobutoxyborane, diphenylboronic anhydride,  
 5 dimethylphenylboronic acid (substitution pattern not  
 established).

Of the compounds listed above those with O-alkyl groups may  
 undergo spontaneous hydrolysis, e.g. diphenylisobutoxyborane,  
 4-(4'-bromophenyl)phenyl-di-n-butoxyborane or 4-bromophenyl-di-  
 10 n-butoxyborane. Others containing the structure (Aryl-O)<sub>2</sub>-B-  
 Aryl may also undergo spontaneous hydrolysis, e.g. di(3',5'-di-  
 chlorophenoxy)-3,5-dichlorophenylborane or 4-chlorophenyl-di-  
 (4'-chlorophenoxy)borane.

The enhancers of the present invention that fall into  
 15 formula (I) may be easily looked at by means of the table below  
 in which H is hydrogen and the key to the compound reference  
 numbers is present in the Examples.

TABLE

		R	W	X	Y	Z
20	5001	H	H	H	bromo	H
	5003	H	H	amino	H	H
	5004	H	H	H	H	H
	5006	H	H	H	iodo	H
	5007	H	H	H	2'-carboxyethenyl	H
25	1001	H	H	H	trimethylsilyl	H
	1003	H	methoxy	H	H	5-bromo
	1005	H	hydroxy	H	H	5-(3'trifluoro methyl)phenylazo
30	1009	H	methyl	H	H	H
	1010	H	H	H	phenoxy	H
	1011	H	H	H	phenyl	H
	1012	O,O-propylene	H	H	H	H
35	1013	1015, 1017, 1018, 1019 and 1048 named compounds				

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	R	W	X	Y	Z
1021	H	H	nitro	chloro	H
1022	H	chloro	chloro	H	H
1024	H	H	chloro	chloro	H
5 1026	H	chloro	amino	chloro	6-chloro
1028	H	H	chloro	H	H
1029	H	H	H	chloro	H
1030	H	H	nitro	H	H
1033	H	H	H	4'-chloroanilino	H
10 1034	H	H	H	methyl	H
1037	H	H	H	4'-boronyl- phenyl	H
1038	n-butyl	H	H	4'-bromo phenyl	H
15 1040	3',5'-dichloro- phenyl	H	chloro	H	5-chloro
1041	4'-chlorophenyl	H	H	chloro	H
1044	n-butyl	H	H	bromo	H
1045	H	H	nitro	carboxy	H

20

1006 fused benzene, hydroxy  
substituted in 6 position  
of naphthalene ring

1002 fused benzene

25 While the invention applies to increasing the light output and/or the signal:background ratio from any chemiluminescent reaction involving the above-stated reaction partners, for any purpose, it is primarily of interest in connection with an assay. The term "assay" herein covers detection, semi- quantitation and

30 quantitation. Typically, the assay is carried out so that the light output is relatable to the amount of peroxidase employed, the peroxidase then being the substance directly determined. The

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ratio of light output when peroxidase is present in the sample to light output when it is absent becomes important in assuring the sensitivity of the assay. This is conveniently termed a "signal to background" ratio. Similarly, if the substance to be determined is another of the reaction partners, the "signal" denotes the presence of the substance to be determined, the "background" its absence.

Although the invention is usable to determine the presence or amount of any one of the four above-stated reaction partners, such a reaction partner is not necessarily itself the substance to be assayed. Thus, the oxidant can be produced as a result of an earlier reaction or cascade of earlier reactions carried out on a sample. The peroxidase or the luminol can be in the form of a conjugate to, say, an antibody which is used in an immunoassay to determine an antigen. The invention is accordingly applicable to any method of diagnostic assay of a substance, the presence or amount of which is relatable to the presence or amount of a reaction partner selected from the group consisting of a DPD, a peroxidase enzyme an oxidant and an enhancer which together are reactable in a chemiluminescent reaction and wherein the reaction is carried out, the light output is detected or measured and thence the presence or amount of the substance to be assayed is related to the light output.

The invention also includes a kit for use in the assay comprising the DPD, the peroxidase and the enhancer. The oxidant could be supplied separately or included in the kit.

#### Brief description of the drawings

The Figure shows the structure of some of the enhancers of the present invention.

#### Description of the preferred embodiments

The enhancers of the present invention include 4-iodophenylboronic acid (PIBA), 4-bromophenylboronic acid (PBBA), 4-chlorophenylboronic acid, 3-chlorophenylboronic acid, 3,4-dichlorophenylboronic acid, 2,3-dichlorophenylboronic acid, 5-bromo-2-methoxybenzeneboronic acid, 3-nitrophenylboronic acid, 4-chloro-



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3-nitrophenylboronic acid, 3-aminophenylboronic acid, 3-amino-2,4,6-trichlorophenylboronic acid, 4-(2'-carboxyethenyl)phenylboronic acid, 1-naphthaleneboronic acid, 6-hydroxy-2-naphthaleneboronic acid, phenylboronic acid, 2-methylphenylboronic acid, 5 4-methylphenylboronic acid, dimethyl-phenylboronic acid, 4-bromophenyl-di-n-butoxyborane, 4-carboxy-3-nitrophenylboronic acid, 4-(trimethylsilyl)benzeneboronic acid, 4-biphenylboronic acid, 4-(phenoxy)benzeneboronic acid, 4-(3'-borono-4'-hydroxyphenylazo)benzoic acid, diphenylisobutoxyborane, 4-(4'chloro-10 anilino)phenylboronic acid, 4,4'-bis(phenylboronic acid), 4-(4'-bromophenyl)phenyl-di-n-butoxyborane, di(3',5'-dichlorophenoxy)-3,5-dichlorophenylborane, 4-chlorophenyl-di-(4'-chlorophenoxy)borane, pentaerythritol borate, boroglycine, 2-phenyl-1,3,2-dioxaborinane, bis(catechol)borate and 2-hydroxy-5-(3'-15 trifluoromethyl)-phenylazo)benzene boronic acid and diphenylboronic anhydride.

The preferred enhancers are PIBA, PBBA, 4-biphenylboronic acid, 4-(trimethylsilyl)-benzeneboronic acid, boroglycine, 2-hydroxy-5-(3'-(trifluoromethyl)phenylazo)benzeneboronic acid, 20 4-chloro-3-nitrophenylboronic acid, 4-chlorophenylboronic acid, 4-(2'-carboxyethenyl)phenylboronic acid, 4-(4'-bromophenyl)phenyl-di-n-butoxyborane, 4-chlorophenyl-di-(4'-chlorophenoxy)borane, 4-4'-bis(phenylboronic acid), diphenylboronic anhydride, 4-chloroanilinophenylboronic acid and 4-bromophenyl-di-n-butoxyborane as 25 they increase light output as well as reducing the background luminescence. The remaining enhancers exert their effect primarily by reducing the background luminescence, and thereby improving the signal:background ratio.

The structure of some of these compounds are shown in the 30 accompanying Figure. The reference numbers are explained in the Examples.

The improvement in signal:background ratio is of importance in controlling the sensitivity of chemiluminescent assays. The enhancers of the present invention are therefore of particular use 35 in those situations where a high degree of sensitivity is

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required, for example in blotting assays. Thus the present invention is of especial use in blotting assays including Western, Southern and Northern blotting assays, as well as dot blots and other nucleic acid hybridisation assays.

- 5       The best results are obtained at higher pH. Preferably the pH is in the range 7.5 to 9 at the time of mixing all the reagents.

- Any chemiluminescent DPD can be used in the invention, that is to say any DPD which is oxidisable in the presence of a peroxidase catalyst by an added oxidant to give chemiluminescence
- 10       can be used. Examples are luminol, isoluminol, ABEI and AHEI, and 7-dimethylaminonaphthalene-1,2-dicarboxylic acid hydrazide, of which luminol is normally preferred. The DPD can be free or conjugated to a ligand to provide a direct label. Such luminophore-labelled assays are known in the art.

- 15       The oxidant can be any added substance (not oxygen itself) which oxidises the DPD in a light-emitting reaction; hydrogen peroxide is usual, but a perborate, such as the sodium salt, is an alternative.

- The peroxidase enzyme will normally be HRP and of a grade
- 20       appropriate to use in luminescent assays. Preferably the HRP is a basic isoenzyme, for example of Sigma Type VIA or IX. It can be free or conjugated to a ligand.

- The concentrations of the reaction partners of the chemiluminescent reaction will depend on the nature of the assay
- 25       being carried out and particularly on which of them is being assayed. Generally stated, the light output is greater, the greater the concentration of DPD. Thus, when peroxidase or oxidant is being assayed, the use of excess DPD is recommended. Generally stated, the DPD concentration is desirably from 0.5
- 30       micromole to 200 millimoles per litre, preferably 0.5 to 100 micromoles/litre. Generally stated, the oxidant concentration is desirably in the range 0.5 micromoles to 300 millimoles/ litre, preferably 10 to 200 millimoles/litre.

- The concentration of peroxidase is of interest if peroxidase
- 35       is not the reaction partner being assayed. Excess peroxidase does

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not normally have a marked effect on light intensity, the peroxidase being a catalyst which is recycled. Where luminol or the oxidant is being assayed, therefore, the peroxidase need only be present in a modest concentration, such as 0.01 microgram to 5000 mg./litre, preferably not more than 50 mg./litre, but depending on the activity of the peroxidase per gram.

The concentration of the enhancer will usually be in the range 0.01 micromole to 4 moles/litre, preferably 10 micromoles to 100 millimoles/litre. It is believed that the enhancer or a species or derivative thereof competes with the DPD in the reaction and it is therefore desirable to use a considerable excess of DPD relative to the enhancer, preferably between 2 and 20 times the molar concentration of the enhancer.

In brief, all conditions and features of the chemiluminescent reactions, the reaction partners thereof, applications of the assay and so on (except where inconsistent with the above description) can be as set forth in European Patent No. 116454B, the disclosure of which in relation thereto is herein incorporated by reference.

The following Examples illustrate the invention. In the Examples, compounds of series 5000 are obtainable from the stated sources, compounds of series 1000 are identified by means of their Chemical Abstracts Registry Number and others are obtainable from US Borax Research Corporation, 412 Crescent Way, Anaheim, California 92901-9794.

#### EXAMPLE 1

This Example shows that para-bromophenylboronic acid (PBBA) enhances a chemiluminescent reaction between luminol (LU) horseradish peroxidase (HRP), and  $H_2O_2$  giving a high signal: background ratio.

#### 1. Effect of PBBA (Compound 5001) on the signal:background ratio of a chemiluminescent reaction

PBBA was added in various concentrations (range 0.05 $\mu$ g to 0.5 $\mu$ g) to a luminol- $H_2O_2$  reaction in the presence and absence of HRP. The luminol-hydrogen peroxide reagent was prepared as follows: sodium luminol (12.5mg) was dissolved in 50ml of Tris

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buffer (0.1mol/l, pH 8.6), and 15.5 $\mu$ l of hydrogen peroxide (30% w/v) was mixed with 0.5ml of Tris buffer (0.1mol/l, pH 8.6). These two solutions were combined and protected from light. The luminol-hydrogen peroxide reagent (100 $\mu$ l of a 1:10 dilution) was added to a cuvette together with either 10 $\mu$ l of HRP Type VI A (HRP, Sigma Chemical Co., 1:50,000 dilution) or as a control 10 $\mu$ l of Tris Buffer (0.1mol/l, pH 8.6), and various amounts of PBBA (Aldrich Chemical Co.), range 0.05 $\mu$ g-0.5 $\mu$ g in 0.1mol/l Tris, pH 8.6). The reagents were mixed and the light emission recorded after 5 minutes on a Berthold Biolumat LB9500C.

This experiment was repeated, replacing the luminol with isoluminol. The measured light output and signal:background ratios are shown in Table 1. Column (a) shows that PBBA enhances the light emission in the luminol-HRP-peroxidase reaction. Column (b) shows that addition of PBBA reduces the background level of luminescence. Columns (d) and (e) show a similar effect with isoluminol.

TABLE 1

PBBA ( $\mu$ g)	Luminol			Isoluminol		
	(a) Signal (with HRP)	(b) Signal (no HRP)	(c) Signal: Background ratio	(d) Signal (with HRP)	(e) Signal (no HRP)	(f) Signal: Background ratio
0	87,616	18,833	4.7	14,485	7927	1.8
0.05	101,227	16,556	6.1	15,772	7664	2.1
0.1	108,213	16,065	6.7	16,553	6114	2.7
0.2	112,644	13,890	8.1	68,282	5736	11.9
0.3	122,116	10,899	11.2	619,880	5613	110.4
0.4	133,401	10,706	12.5	775,965	5278	147.0
0.5	151,572	8,963	16.9			

## 2. Effect of PBBA on the light output of a chemiluminescent reaction

The above experiment was repeated this time keeping the amount of PBBA enhancer fixed and varying the amount of HRP. The reagents used were 100 $\mu$ l of luminol-hydrogen peroxide, either (a) 40 $\mu$ l of PBBA (0.01mg/ml in 0.1mol/l Tris buffer,

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pH 8.6) or (b) as a control, 40 $\mu$ l of Tris buffer (0.1mol/l, pH 8.6) and various amounts of HRP (range 5 $\mu$ l to 40 $\mu$ l of 1:50,000 dilution of a 1mg/ml stock solution).

The reagents were mixed and light output recorded after 5 minutes on a Berthold Biolumat LB9500C. The measured signal (light output) and signal:background ratios are shown in Table 2. By comparing columns (a) and (b) it can be seen that PBBA increased the light output of the chemiluminescent reaction at all concentrations tested. An improved signal:background ratio is shown by comparing results with HRP present with the zero HRP results.

TABLE 2

HRP (pg)	(a) Signal (with PBBA)	Signal: Background Ratio	(b) Signal (no PBBA)	Signal: Background Ratio
0	9,125		13,405	
100	15,861	1.7	15,148	1.1
200	22,895	2.5	19,384	1.4
400	38,495	4.2	25,968	1.9
600	61,491	6.7	33,964	2.5
800	105,972	11.6	34,790	2.6

EXAMPLE 2Effect of organoboron compounds on a chemiluminescent reaction

A solution of 2,4-dichlorophenylboronic acid (5002) (1mg/ml, Lancaster Synthesis Inc., Windham, NH) was prepared as follows: 5mg 2,4-dichlorophenylboronic acid was dissolved in 50 $\mu$ l of DMSO, then added to 4950 $\mu$ l of Tris buffer (0.1mol/l, pH 8.6). Solutions of 3-aminophenylboronic acid (5003) (1mg/ml, Sigma), 3-nitro-phenylboronic acid (1mg/ml, Aldrich), phenylboronic acid (5004) (1mg/ml, Aldrich) and butaneborinic acid (5005) (1mg/ml, Sigma) were prepared in Tris buffer (0.1mol/l, pH 8.6). The stock solution of HRP Type VIA (1mg/ml) and luminol-hydrogen peroxide reagent were prepared as described previously. Luminol-hydrogen peroxide (100 $\mu$ l, 1:10 dilution) was mixed with either 10 $\mu$ l of HRP (1:50,000 dilution) or, 10 $\mu$ l of Tris buffer

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(0.1mol/l, pH 8.6). The light emission was measured in a Berthold Biolumat LB9500C, and then 5 $\mu$ l of 2,4-dichlorophenylboronic acid was added and the light emission was remeasured. A control without any test compound was run in parallel.

- 5       The above experiment was repeated except that 2,4-dichlorophenylboronic acid was replaced by 3-aminophenylboronic acid (5 $\mu$ l), 3-nitrophenylboronic acid (5 $\mu$ l), phenylboronic acid (5 $\mu$ l), or butaneborinic acid (40 $\mu$ l).

- 10       The measured light output and signal:background ratios are shown in Table 3 for each compound tested and its control. The control consisted of buffer in place of the compound under test as an enhancer. None of the compounds tested increased the light emission from the HRP catalyzed oxidation of luminol. However, the signal to background ratio was improved in the case  
15       of 3-nitrophenylboronic acid, phenylboronic acid, and 3-aminophenylboronic acid as compared to their control values. This was due to the reduction in the background light emission from the luminol-peroxide reagent caused by these compounds. Thus, these three compounds are of use in the present invention. 2-4-  
20       dichlorophenylboronic acid and butane boronic acid are not of use as they neither increased the light output or the signal:background ratio. The small increase in light output observed for butaneborinic acid is not significantly relevant to be of use in the present invention.

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TABLE 3

5			Signal (with HRP	Signal (no HRP	Signal: Background Ratio
10	2,4-dichlorophenyl- boronic acid (5002)	control	271,566	20,990	12.9
		test	128,274	16,246	7.9
15	3-nitrophenyl- boronic acid	control	175,440	25,196	6.9
		test	132,887	16,400	8.1
20	phenylboronic acid (5004)	control	156,658	26,564	5.9
		test	54,554	5,601	9.7
25	3-aminophenyl- boronic acid (5003)	control	249,204	19,615	12.7
		test	60,222	1,038	58.0
30	butaneborinic acid (5005)	control	64,652	39,640	1.6
		test	65,197	54,389	1.2

EXAMPLE 325 Screening of further organoboron compounds

The stock solution of HRP Type VIA (1mg/ml) and luminol-hydrogen peroxide were prepared as described above. 10µl test compound of varying concentration (0.01-1mg/ml) was added to luminol-hydrogen peroxide (100µl, 1:10 dilution). 10µl HRP Type VIA or as a control 10µl Tris buffer was added. The light emission was measured on a Berthold Biolumat LB9500C.

The following test compounds were all screened in this manner, Chemical Abstracts Registry Numbers are in brackets:

- 1001 4-(trimethylsilyl) benzeneboronic acid (17865-11-1)
- 35 1002 1-naphthaleneboronic acid (31093-44-4)
- 1003 5-bromo-2-methoxybenzeneboronic acid (84694-45-1)
- 1004 2-biphenylboronic acid (4688-76-0)
- 1005 2-hydroxy-5-(3'-(trifluoromethyl)-phenylazo)benzeneboronic acid
- 40 1006 6-hydroxy-2-naphthalene boronic acid
- 1007 1-thianthreneboronic acid (108847-76-3)
- 1008 4-dibenzofuranboronic acid (100124-06-9)
- 1009 2-tolueneboronic acid (16419-60-6)

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- 1010 4-(phenoxy)benzeneboronic acid (109412-50-2)  
1011 4-biphenylboronic acid (5122-94-1)  
1012 2-phenyl-1,3,2-dioxaborinane (4406-77-3)  
1013 bis(catechol)borate  
5 1014 boraxarophenanthrene  
1015 boroglycine  
1016 tetraphenylboron sodium (143-66-8)  
1017 pentaerythritol borate  
1018 4-(3'-borono-4'-hydroxyphenylazo)benzoic acid  
10 1019 diphenylisobutoxyborane (23147-97-9)  
1020 2,4,6-trichlorophenylboronic acid (73852-18-3)  
1021 4-chloro-3-nitrophenylboronic acid  
1022 2,3-dichlorophenylboronic acid  
1023 2,5-dichlorophenylboronic acid (135145-90-3)  
15 1024 3,4-dichlorophenylboronic acid  
1025 3,5-dichlorophenylboronic acid (67492-50-6)  
1026 3-amino-2,4,6-trichlorophenylboronic acid  
1027 2-chlorophenylboronic acid  
1028 3-chlorophenylboronic acid (63503-60-6)  
20 1029 4-chlorophenylboronic acid (1679-18-1)  
1030 3-nitrophenylboronic acid (13331-27-6)  
1031 3-chloroacetylaminophenylboronic acid  
1032 3-(2'-methylbutylamino)phenylboronic acid  
1033 4-(4'-chloroanilino)phenylboronic acid  
25 1034 4-methylphenylboronic acid (5720-05-8)  
1035 1,4-phenyldiboronic acid  
1036 dimethylphenylboronic acid (position of methyl groups  
not established)  
1037 4,4'-bis(phenylboronic acid) (4151-80-8)  
30 1038 4-(4'-bromophenyl)phenyl-di-n-butoxyborane  
1039 di-(3',4',6'-trichlorophenoxy)-3,4,6-trichlorophenyl-  
borane  
1040 di-(3',5'-dichlorophenoxy)-3,5-dichlorophenylborane  
1041 4-chlorophenyl-di-(4'-chlorophenoxy)borane  
35 1042 3-nitrophenylboronic acid, sodium salt  
1043 3-nitrophenylboronic acid, calcium salt



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- 1044 4-bromophenyl-di-n-butoxyborane
- 1045 4-carboxy-3-nitrophenylboronic acid
- 1046 2-benzimidazolylphenylboronic acid (58534-74-0)
- 1047 di-(1-naphthoxy)-1-naphthylborane
- 5 1048 diphenylboronic anhydride
- 1049 2-boromethylphenyl-di-(2'-boromethylphenoxy)borane
- 1050 2-(methylthiomethyl)phenylboronic acid
- 1051 methyl-(2-tolylboronic acid)sulfoxide

In Table 4, column (a) shows whether the test compound lowered the light output from the luminol-hydrogen peroxide solution (Y = yes, N = no). Column (b) shows whether the signal obtained after adding the HRP was greater in the presence of the test compound than in its absence, (Y = yes, N = no). A compound showing Y in column (b) is of preferred use in the present invention as it increases the light output of the reaction. Column (c) shows whether there was an increase in the signal:background ratio by over 25% of the control value (Y = yes, N = no), column (d) shows the actual calculated value of the signal:background ratio (value of column (b)/value of column (a)). Column (c) is a simplified representation of the results in column (d). Signal:background ratio is the ratio of light output in the presence and absence of HRP with the test compound being present.

All compounds increasing the signal:background ratio by over 25% of at least one concentration (Y in column (c)) are of use in the present invention. Those compounds that increase the light output (Y in column (b)) as well as increasing the signal:background ratio (Y in column (c)) are of especial use in the present invention. Thus, compounds 1001, 1002, 1003, 1005, 1006, 1009, 1010, 1011, 1012, 1013, 1015, 1017, 1018, 1019, 1021, 1022, 1024, 1026, 1028, 1029, 1030, 1033, 1034, 1036, 1037, 1038, 1040, 1041, 1044, 1045 and 1048 are of use in increasing the signal:background ratio of a chemiluminescent reaction. Of these, compounds 1001, 1005, 1011, 1015, 1021, 1029, 1033, 1037, 1038, 1041, 1044 and 1048 also increase the light output and are therefore of especial use in the present invention.

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TABLE 4

5	Compound	mg/ml	(a) Decrease in background (without HRP with test cpd.)	(b) Increase in signal (with HRP, with test cpd.)	(c) Increase in signal: background ratio	(d) Signal: Background: ratio (control = with no enhancer)
10						
	1001	1	Y	N	Y	10.9 (2.1)
		0.1	Y	Y	Y	6.7
		0.01	Y	Y	Y	3.2
15						
	1002	1	Y	N	N	0.7 (2.3)
		0.1	Y	N	Y	3.4
		0.01	Y	N	N	2.5
20						
	1003	1	Y	N	N	2.6 (2.3)
		0.1	Y	N	Y	12.1
		0.01	Y	N	Y	8.6
25						
	1004	1	Y	N	N	1.7 (2.3)
		0.1	Y	N	N	2.2
		0.01	Y	N	N	1.9
30						
	1005	1	Y	N	N	2.4 (2.1)
		0.1	N	Y	Y	2.9
		0.01	N	Y	Y	2.7
35						
	1006	1	Y	N	N	0.3 (1.8)
		0.1	Y	N	N	1.5
		0.01	Y	N	Y	5.8
40						
	1007	1	Y	N	N	1.8 (2.1)
		0.1	Y	N	N	2.5
		0.01	Y	N	N	2.1
45						
	1008	1	Y	N	N	1.3 (2.5)
		0.1	Y	N	N	1.5
		0.01	Y	N	N	1.6
50						
	1009	1	Y	N	N	0.6 (1.8)
		0.1	Y	N	Y	2.5
		0.01	Y	N	N	1.9
	1010	1	Y	N	Y	5.0 (2.5)
		0.1	Y	N	Y	4.6
		0.01	Y	N	N	3.0

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			(a)	(b)	(c)	(d)
5	1011	1	Y	Y	Y	48.7 (2.5)
		0.1	Y	Y	Y	>125
		0.01	Y	Y	Y	>125
10	1012	1	Y	N	Y	17.1 (2.5)
		0.1	N	N	N	2.2
		0.01	N	Y	N	2.2
15	1013	1	Y	N	N	1.2 (1.8)
		0.1	Y	N	Y	3.1
		0.01	Y	N	Y	12.6
20	1014	1	Y	N	N	1.8 (2.5)
		0.1	Y	N	N	1.6
		0.01	Y	N	N	2.3
25	1015	1	Y	N	Y	3.1 (2.1)
		0.1	Y	Y	Y	2.9
		0.01	Y	Y	N	2.3
30	1016	1	Y	N	N	2.3 (1.9)
		0.1	Y	N	N	2.0
		0.01	Y	N	N	2.0
35	1017	1	Y	N	N	1.8 (1.8)
		0.1	Y	Y	Y	2.5
		0.01	Y	N	N	1.8
40	1018	1	Y	N	Y	4.2 (1.9)
		0.1	Y	N	Y	24.5
		0.01	Y	N	Y	5.0
45	1019	1	Y	N	N	
		0.1	Y	N	N	3.0 (2.9)
		0.01	Y	N	Y	3.2 (2.0)
50	1020	1	Y	N	N	
		0.1	Y	N	N	
		0.01	Y	N	N	2.2 (2.0)
	1021	1	Y	Y	Y	247.9 (4.5)
		0.1	Y	Y	Y	44.5 (2.8)
		0.01	Y	Y	Y	2.3 (1.8)
	1022	1	Y	N	N	
		0.1	Y	N	Y	3.6 (2.7)
		0.01	Y	N	N	2.2 (2.0)

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			(a)	(b)	(c)	(d)
5	1023	1	Y	N	N	
		0.1	Y	N	N	3.1 (2.7)
		0.01	Y	N	N	
10	1024	1	Y	N	Y	7.6 (4.5)
		0.1	Y	Y	Y	5.4 (2.8)
		0.01	Y	N	N	
15	1025	1	Y	N	N	
		0.1	Y	N	N	
		0.01	Y	N	N	2.1 (2.0)
20	1026	1	Y	N	Y	40.5 (4.5)
		0.1	Y	Y	Y	72.4 (2.8)
		0.01	Y	N	N	2.1 (2.0)
25	1027	1	Y	N	N	
		0.1	Y	N	N	3.2 (2.9)
		0.01	Y	N	N	2.1 (2.0)
30	1028	1	Y	N	Y	20.0 (4.5)
		0.1	Y	Y	Y	4.2 (2.9)
		0.01	Y	N	N	
35	1029	1	Y	Y	Y	1242.4 (4.5)
		0.1	Y	Y	Y	315.0 (2.8)
		0.01	Y	Y	Y	6.0 (1.8)
40	1030	1	Y	N	Y	50.0 (4.5)
		0.1	Y	Y	Y	12.5 (2.8)
		0.01	Y	N	N	
45	1031	1	Y	N	N	
		0.1	Y	N	N	3.3 (2.9)
		0.01	Y	N	N	2.1 (2.0)
50	1032	1	Y	N	N	
		0.1	Y	N	N	3.4 (2.9)
		0.01	Y	N	N	
50	1033	1	Y	Y	Y	173.9 (4.5)
		0.1	Y	Y	Y	67.7 (2.8)
		0.01	Y	Y	Y	4.1 (1.8)
50	1034	1	Y	N	N	
		0.1	Y	Y	Y	41.2 (2.9)
		0.01	Y	N	Y	3.6 (1.9)

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			(a)	(b)	(c)	(d)
5	1035	1	Y	N	N	
		0.1	Y	N	N	
		0.01	Y	N	N	2.1 (2.0)
10	1036	1	Y	N	N	
		0.1	Y	N	Y	7.6 (2.9)
		0.01	Y	N	Y	2.6 (2.0)
15	1037	1	Y	N	N	
		0.1	Y	Y	Y	503.3 (2.9)
		0.01	Y	Y	Y	35.5 (1.9)
20	1038	1	Y	Y	Y	903.8 (4.5)
		0.1	Y	Y	Y	2118.5 (2.9)
		0.01	Y	Y	Y	442.2 (1.8)
25	1039	1	Y	N	N	
		0.1	Y	N	N	3.4 (2.9)
		0.01	Y	N	N	
30	1040	1	Y	N	Y	4.7 (4.5)
		0.1	Y	N	Y	3.9 (2.9)
		0.01	Y	N	N	
35	1041	1	Y	Y	Y	2606.2 (4.5)
		0.1	Y	Y	Y	360.2 (2.8)
		0.01	Y	Y	Y	6.3 (1.9)
40	1042	1	Y	N	N	5.2 (4.5)
		0.1	Y	N	N	3.6 (2.9)
		0.01	Y	N	N	2.3 (2.0)
45	1043	1	Y	N	N	
		0.1	Y	N	N	3.4 (2.9)
		0.01	Y	N	N	
50	1044	1	Y	N	Y	147.1 (4.5)
		0.1	Y	Y	Y	623.1 (2.7)
		0.01	Y	Y	Y	38.5 (1.9)
50	1045	1	Y	N	Y	68.5 (4.5)
		0.1	Y	Y	Y	18.1 (2.8)
		0.01	Y	N	N	2.2 (2.0)
50	1046	1	Y	N	N	
		0.1	Y	N	N	
		0.01	Y	N	N	

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			(a)	(b)	(c)	(d)
5	1047	1	Y	N	N	
		0.1	Y	N	N	
		0.01	Y	N	N	
10	1048	1	Y	N	Y	100.0 (4.5)
		0.1	Y	Y	Y	139.0 (2.7)
		0.01	Y	Y	Y	3.1 (1.8)
15	1049	1	Y	N	N	
		0.1	Y	N	N	3.2 (2.9)
		0.01	Y	N	N	
20	1050	1	Y	N	N	
		0.1	Y	N	N	3.3 (2.9)
		0.01	Y	N	N	
20	1051	1	Y	N	N	
		0.1	Y	N	N	
		0.01	Y	N	N	

EXAMPLE 4

25 Effect of different peroxidase enzymes on the PBBA enhancement of a chemiluminescent reaction

Stock solutions (1mg/ml in 0.1mol/l Tris buffer, pH 8.6) of horseradish peroxidase Type VII, Type VIII and Arthromyces ramosus peroxidase (Sigma) were prepared. The luminol-hydrogen peroxide reagent was prepared as described previously. Luminol-hydrogen peroxide reagent (100μl, 1:10 dilution), either 40μl of PBBA (0.01mg/ml in 0.1mol/l Tris buffer, pH 8.6) or as a control; 40μl of Tris buffer (0.1mol/l, pH 8.6), and 10μl of HRP Type VII (1:50,000 dilution, 2ng) were added to a cuvette. The reagents were mixed and the light emission was recorded after 5 minutes using a Berthold Biolumat LB9500C.

The above experiment was repeated and the HRP Type VII was replaced by HRP Type VIII (2ng) or Arthromyces ramosus peroxidase (20pg).

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The measured light output and signal:background ratios are shown in Table 5. PBBA did not increase the level of light output with any of the peroxidases tested, but the signal to background ratio was improved in all cases due to the background reduction caused by PBBA.

TABLE 5

	Signal Reagent with PBBA	Signal: Background	Signal Reagent no PBBA	Signal: Background
Blank	27,820		58,759	
HRP Type VII	49,703	1.8	77,694	1.3
HRP Type VIII	156,204	5.6	280,472	4.8
<u>Arthromyces</u> <u>rasmusus</u>	58,208	2.1	84,071	1.4

EXAMPLE 525 Assay of peroxidase-antibody conjugate utilizing PBBA

Anti-mouse IgG (whole molecule) peroxidase conjugate (Sigma Chemical Co.) was diluted (1:5000) in 0.1 mol/l Tris buffer (pH 8.6). The luminol-hydrogen peroxide was prepared as described previously. The following reagents were added to a cuvette: 100µl of luminol-hydrogen peroxide (1:10 dilution), either 40µl of PBBA (0.01 mg/ml in 0.1 mol/l Tris buffer) or as a control, 40µl of Tris buffer (0.1 mol/l, pH 8.6), and different amounts of anti-mouse IgG (5µl, 10µl, 20µl, 30µl, 40µl). The reagents were mixed and the light emission was measured using a Berthold Biolumat LB9500C.

The results are shown in Table 6. In the presence of PBBA the light emission was increased and the signal to background ratio for the assay of the HRP conjugate was significantly improved.

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TABLE 6

	Anti-mouse IgG-HRP (ml)	Signal with PBBA	Signal: Background	Signal no PBBA	Signal: Background
5	0	18,832		23,112	
	5	27,665	1.5	17,858	0.8
10	10	32,849	1.7	27,458	1.2
	20	68,994	3.7	29,850	1.3
	30	407,087	21.6	32,417	1.4

15 EXAMPLE 6Enhanced chemiluminescent anti-oxidant assay using PBBA

The luminol-peroxide reagent (100 $\mu$ l), PBBA (40 $\mu$ l, 1:100 dilution of 1 mg/ml) and HRP (10 $\mu$ l, 1:50,000 dilution of 1mg/ml stock) were mixed together and the light emission measured for 20 25 minutes. A sample of human serum (2 $\mu$ l, 1:10 dilution in 0.1 mol/l Tris buffer, pH 8.6) was added and the light emission measured for a further 75 minutes.

Table 7 shows that addition of serum quenched the light emission and that after a 34 minute lag the light emission 25 returns to its original level.

TABLE 7

	Time (minutes)	Light units	Time (minutes)	Light units
30	0	5	50	5
	10	16	60	15
	20	68	70	49
35	(serum added) → 25	88	80	60
	26	3	90	81
	30	3	100	90

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EXAMPLE 7Effect of para-iodophenylboronic acid (PIBA) (5006) as an enhancer of a chemiluminescent reaction

The experiment of Example 1 was repeated using PIBA in place of PBBA. Various dilutions of PIBA (Cookson Chemicals Ltd., Southampton, UK, 1mg/ml stock in DMSO) in Tris buffer were used. Table 8 shows the effect PIBA has on the signal:background ratio of a chemiluminescent reaction measured after 1 minute.

TABLE 8

4-Iodophenylboronic acid (PIBA) enhancement of the  
HRP-luminol-peroxide reaction

	PIBA	Light emission at 1 minute		Signal:Background Ratio
	µg	Background	Signal	
20	0	15,016	91,665	6.1
	0.01	12,862	78,419	6.1
	0.1	13,221	74,487	7.1
	1	10,282	324,312	31.5
	10	5,517	540,760	98
25	20	3,837	135,065	35.2

EXAMPLE 8Use of an organoboron enhancer in a chemiluminescent assay for

30 a) peroxide and b) luminol

a) Effect of 4-biphenylboronic acid on a chemiluminescent assay  
for peroxide

The assay reagent consisted of the following reagents: 10µl 4-biphenylboronic acid (1:20 dilution of a 1mg/ml stock in DMSO), 10µl horseradish peroxidase (1:500,000 dilution of a 1mg/ml stock Type VIA in 0.1 mol/l Tris buffer pH 8.6) and 100µl luminol (0.025 g/l in 0.1 mol/l Tris buffer pH 8.6). These were mixed together and the light emission measured. A 10µl sample of a hydrogen peroxide solution (dilutions of a stock, 31µl 30% w/v

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hydrogen peroxide/ml in Tris buffer) was added, the contents of the assay tube mixed and the light emission recorded. The results are summarised in Table 9.

From Table 9 it can be seen that there was a dose-dependent increase in light output up to 2.4  $\mu$ moles of peroxide.

TABLE 9Enhanced chemiluminescent assay for peroxide

	Hydrogen peroxide $\mu$ moles	Light emission at 5 minutes
	0	525
	0.00024	659
	0.0024	1,500
	0.024	40,682
	0.24	584,638
	2.4	620,551
	24	339,291

b) Effect of 4-biphenylboronic acid on a chemiluminescent assay for luminol

The assay reagent consisted of the following reagents: 10  $\mu$ l 4-biphenylboronic acid (1:20 dilution of a 1mg/ml stock in DMSO), 10  $\mu$ l horseradish peroxidase (1:500,000 dilution of a 1mg/ml stock Type VIA in 0.1 mol/l Tris buffer pH 8.6) and 100  $\mu$ l hydrogen peroxide solution (31  $\mu$ l 30% w/v hydrogen peroxide diluted in 100ml Tris buffer). These were mixed together and the light emission measured. A 10  $\mu$ l sample of a luminol solution (dilutions of a 0.25g/l in 0.1 mol/l Tris buffer pH 8.6 stock) was added, the contents of the assay tube mixed and the light emission recorded. The results are summarised in Table 10.

From Table 10 it can be seen that there was a dose-dependent increase in light output at all concentrations of luminol tested.

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TABLE 10

Enhanced chemiluminescent assay for luminol

5	Luminol μmoles	Light emission at 10 minutes
	0	54
10	0.00125	145
	0.0125	620
	0.125	5,161
	1.25	55,873
	12.5	358,184
15	125	834,089

EXAMPLE 9

20 Effect of 4-(2-carboxyethenyl)phenylboronic acid (CPA) (5007) as  
an enhancer of a chemiluminescent reaction

The experiment of Example 1 was repeated using CPA in place  
of PBBA. Various dilutions of CPA (Cookson Chemicals Ltd.,  
Southampton, UK, 0.01-20 μg in 1 mg/ml Tris buffer, pH 8.6) were  
used. Table 11 shows the effect CPA had on the light output  
25 (signal) and signal to background ratio of a chemiluminescent  
reaction measured using an Amerlite microplate reader  
(Kodak Clinical Diagnostics, Amersham, UK).

TABLE 11

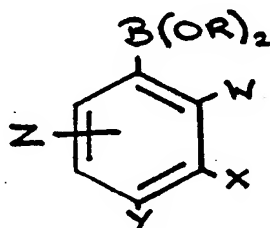
30	CPA (μg)	Signal (with HRP)	Signal (no HRP)	Signal: Background ratio
35	0	0.37	0.088	4.2
	0.01	0.41	0.094	4.4
	0.02	0.46	0.09	5.1
	0.05	0.68	0.09	7.6
40	0.1	1.23	0.09	14.2
	0.2	2.37	0.08	28.2
	0.5	4.57	0.07	63.4
	1.0	5.58	0.06	93.0
	10	2.49	0.02	124.9
45	20	0.88	0.003	43.9

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CLAIMS

1. A method of increasing the light output and/or signal: background ratio of light output from a chemiluminescent reaction of a dihydrophthalazinedione (DPD), a peroxidase enzyme catalyst and an oxidant, by carrying out this reaction in the presence of an enhancer, "signal" being in the presence of the peroxidase, "background" in its absence, characterised in that the enhancer includes compounds of formula (I)

10



(I)

in which R, W, X, Y and Z have the following meanings;

- 15 R is selected from hydrogen, n-butyl, O,O-propylene (a cyclic ether), 4'-chlorophenyl and 3',5'-dichlorophenyl, W is selected from hydrogen, hydroxy, methyl, methoxy and chloro, X is selected from hydrogen, methyl, chloro, amino and nitro, Y is selected from hydrogen, methyl, carboxy, chloro, bromo, iodo, phenyl, phenoxy, 4'-chloroanilino, 4'-boronylphenyl, 4'-bromophenyl, 2'-carboxyethenyl and trimethylsilyl, Z is selected from hydrogen, 5-chloro, 5-bromo, 5-(3'-trifluoromethyl)phenylazo, or 6-chloro, W and X together may represent a fused benzene ring and X and Y together may represent a fused benzene ring substituted by hydroxy in the 6 position of the naphthalene ring numbering, provided that
- 25 when R is hydrogen, W, X, Y, Z are each separately hydrogen;  
 when R, W, X and Z are each hydrogen Y is selected from iodo, bromo, chloro, trimethylsilyl, phenoxy, phenyl, 4'-chloroanilino, methyl, 4'-boronylphenyl, 2'-carboxyethenyl;
- 30 when Y is hydrogen and the Rs together represent O,O-propylene (a cyclic ether), X is hydrogen;

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- when R, W and Z are each hydrogen, X and Y together represent a fused benzene ring substituted by hydroxy in the 6-position of the naphthalene ring numbering;
- when W, X and Z are each hydrogen, R is n-butyl and Y is bromo or 4'-bromophenyl;
- 5 when W, X and Z are each hydrogen, R is 4-chlorophenyl and Y is chloro;
- when W and Y are each hydrogen, R is 3',5'-dichlorophenyl, Y is chloro and Z is 5-chloro;
- 10 when W is methoxy, Z is 5-bromo;
- when W is hydroxy, Z is 5-(3'-trifluoromethyl)phenylazo;
- when W is chloro, X is chloro;
- when Y is chloro, X is nitro or chloro;
- when Y is carboxy, X is nitro;
- 15 when W and Y are each chloro and X is amino, Z is 6-chloro;
- and the compounds bis(catechol)borate, boroglycine, pentaerythritol borate, 4-(3'-borono-4'-hydroxyphenylazo)benzoic acid, diphenylisobutoxyborane, diphenylboronic anhydride, dimethylphenylboronic acid (substitution pattern not
- 20 established) and the sodium salt of 3-nitrophenylboronic acid.
2. A method according to Claim 1, characterised in that the enhancer is para-iodophenylboronic acid, para-bromophenylboronic acid, 4-bi-phenylboronic acid, 4-(trimethylsilyl)benzeneboronic acid, 2-hydroxy-5-(3'-(trifluoromethyl)phenylazo)benzeneboronic
- 25 acid, boroglycine, 4-chloro-3-nitrophenylboronic acid, 4-chlorophenyl-boronic acid, 4-(2'-carboxyethenyl)phenylboronic acid, 4-(4'-bromophenyl)phenyl-di-n-butoxyborane, 4-chlorophenyl-di-(4'-chlorophenoxy)borane, 4-4'-bis(phenylboronic acid), diphenylboronic anhydride, 4-chloroanilinophenylboronic acid or
- 30 4-bromo-phenyl-di-n-butoxyborane.
3. A method according to any preceding claim wherein the peroxidase enzyme is free or conjugated to a ligand and the presence or amount of the peroxidase is determined from the presence or amount of light output.

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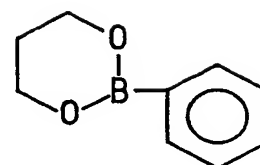
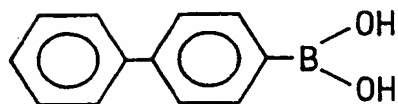
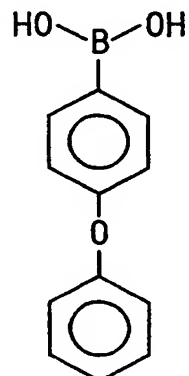
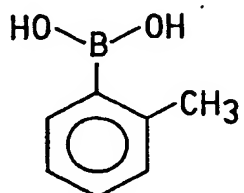
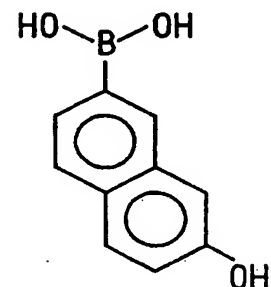
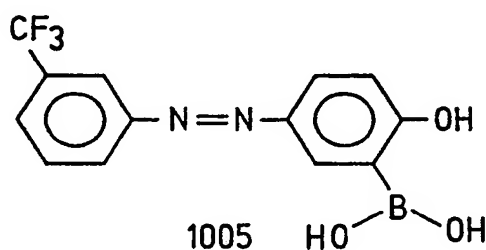
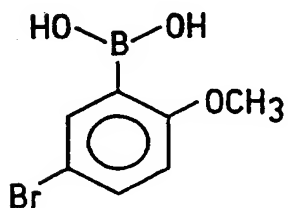
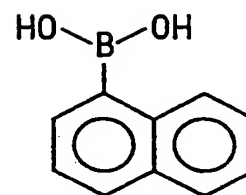
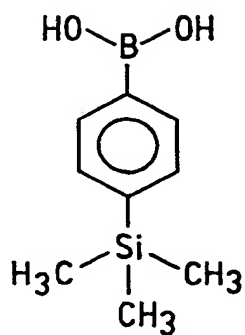
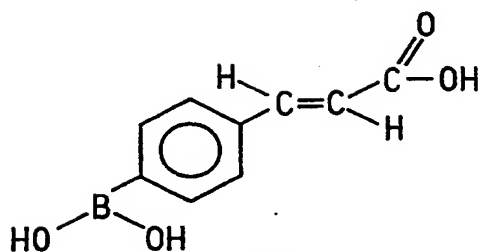
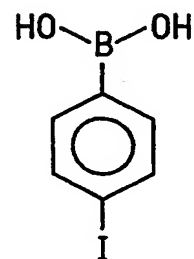
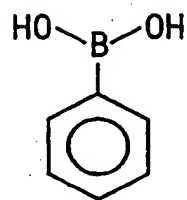
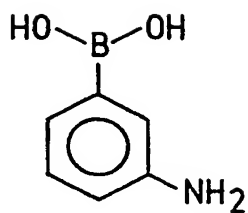
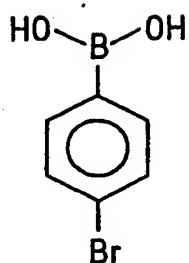
4. A method according to any preceding claim wherein the peroxidase is horseradish peroxidase.
5. A method according to any preceding claim wherein the oxidant is hydrogen peroxide.
- 5 6. A method according to any preceding claim wherein the DPD is luminol.
7. A method according to any preceding claim wherein the chemiluminescent reaction is carried out at a pH of from 7.5 to 9.
8. A method according to any preceding claim for use in  
10 diagnostic assay for peroxidase.
9. A kit for use in diagnostic assay comprising in separate containers :
  - a chemiluminescent dihydrophthalazinedione (DPD);
  - a peroxidase enzyme catalyst; and
  - 15 an enhancer which increases the signal:background ratio of light output, "signal" being in the presence of the peroxidase, "background" in its absence, characterised in that the enhancer is any one of the enhancers of Claim 1.
- 20 10. A kit according to Claim 9 wherein the enhancer is para-iodophenylboronic acid, para-bromophenylboronic acid, 4-biphenylboronic acid, 4-(trimethylsilyl)benzeneboronic acid, 2-hydroxy-5-(3'-(trifluoromethyl)phenylazo)benzeneboronic acid, boroglycine, 4-chloro-3-nitrophenylboronic acid, 4-chlorophenyl-  
25 boronic acid, 4-(2'-carboxyethenyl)phenylboronic acid, 4-(4'-bromophenyl)phenyl-di-n-butoxyborane, 4-chlorophenyl-di-(4'-chlorophenoxy)borane, 4-4'-bis(phenylboronic acid), diphenylboronic anhydride, 4-chloroanilinophenylboronic acid and 4-bromophenyl-di-n-butoxyborane.
- 30 11. A kit according to Claim 9 or 10 wherein the peroxidase is conjugated to a ligand.
12. A kit according to Claim 9, 10 or 11 wherein the peroxidase is horseradish peroxidase.
13. A kit according to any one of Claims 9 to 12 which further  
35 comprises an oxidant.

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14. A kit according to Claim 13 wherein the oxidant is hydrogen peroxide.

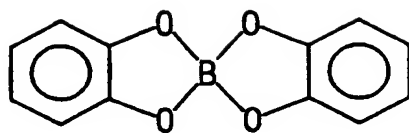
15. A kit according to any one of Claims 9 to 14 wherein the DPD is luminol.

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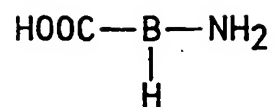




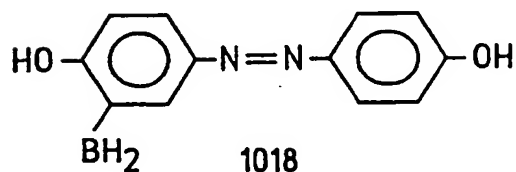
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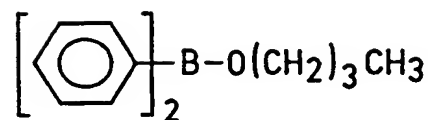
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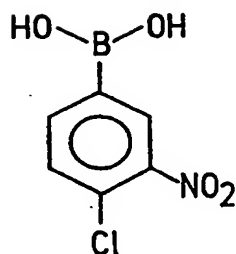
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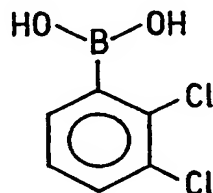
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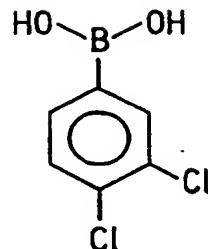
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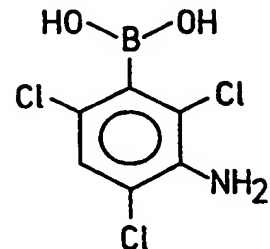
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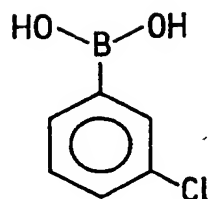
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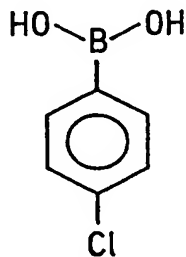
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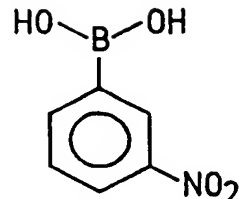
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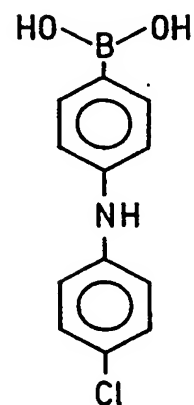
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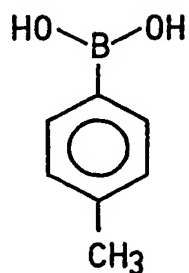


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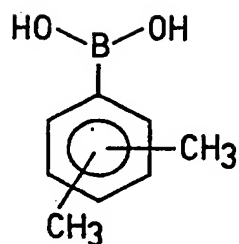


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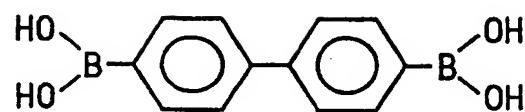
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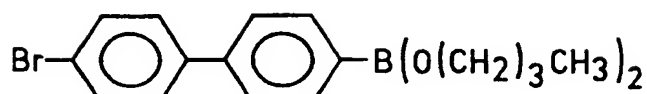
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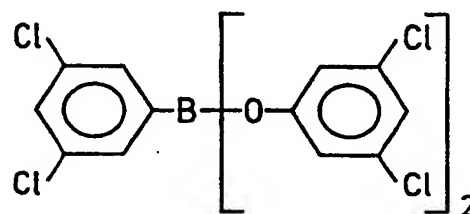
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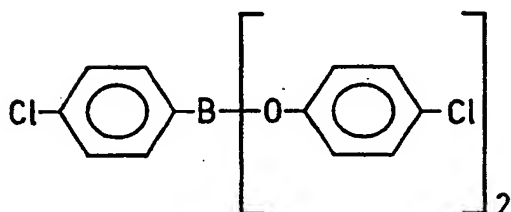
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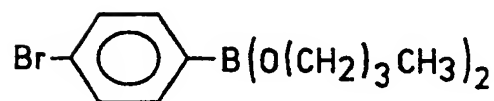
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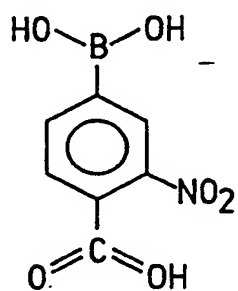
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## INTERNATIONAL SEARCH REPORT

International Application

PCT/GB 93/00271

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 C12Q1/28		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
Int.Cl. 5	C12Q ; G01N	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>		
Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
A	METHODS IN ENZYMOLOGY vol. 133, 1986, NEW YORK (US) pages 331 - 353 GARY H. THORPE AND LARRY J. KRICKA 'Enhanced Chemiluminescent Reactions Catalyzed by Horseradish Peroxidase' see the whole document ---	1-15
A	CLINICAL CHEMISTRY. vol. 37, no. 9, September 1991, WINSTON US pages 1472 - 1481 LARRY J. KRICKA 'Chemiluminescent and Bioluminescent Techniques' cited in the application see page 1477, right column, line 9 - line 36; table 3 --- -/--	1-15
<p><sup>10</sup> Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
28 MAY 1993		17.06.93
International Searching Authority		Signature of Authorized Officer
EUROPEAN PATENT OFFICE		DÖPFER K.P.

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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category <sup>a</sup>	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	GB,A,2 162 946 (NATIONAL RESEARCH DEVELOPMENT CORPORATION (UNITED KINGDOM)) 12 February 1986 cited in the application see the whole document ----	1-15
A	WO,A,9 105 872 (NATIONAL RESEARCH DEVELOPMENT CORPORATION) 2 May 1991 see page 2, line 16 - page 4, line 15; claims 1-10; example 15 -----	1-15

**ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.**

**GB 9300271  
SA 69876**

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.  
The members are as contained in the European Patent Office EDP file on  
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

**28/05/93**

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
GB-A-2162946	12-02-86	DE-A- 3528391	20-02-86
		JP-A- 61054453	18-03-86
		US-A- 4729950	08-03-88
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WO-A-9105872	02-05-91	EP-A- 0496793	05-08-92
		GB-A- 2237383	01-05-91
		JP-T- 5501203	11-03-93
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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

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